# Genetic selection for fast growth generates bone architecture characterised by enhanced periosteal expansion and limited consolidation of the cortices but a diminution in the early responses to mechanical loading.

# GENETIC SELECTION FOR FAST GROWTH GENERATES BONE ARCHITECTURE CHARACTERISED BY ENHANCED PERIOSTEAL EXPANSION AND LIMITED CONSOLIDATION OF THE CORTICES BUT A DIMINUTION IN THE EARLY RESPONSES TO MECHANICAL LOADING

Simon CF Rawlinson[1, 7], Dianne H Murray[2, 7], John R Mosley[3], Chris DP Wright[4], John C Bredl[4], Leanne K Saxon[4], Nigel Loveridge[5], Christine Leterrier[6], Paul Constantin[6], Colin Farquharson[2, 8] and Andrew A Pitsillides[4, 8]

[1]Centre for Adult Oral Health, Bart's and The London School of Medicine and Dentistry, Queen Mary's University of London, UK.
[2]Division of Developmental Biology, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, UK.
[3]Department of Veterinary Clinical Studies. The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK.
[4]Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, London, UK.
[5]Bone Research Group, Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK.
[6]INRA-CNRS (UMR 6175)-Université François Rabelais de Tours-Haras nationaux, Unité de Physiologie de la Reproduction et des Comportements, INRA- Centre de Tours-Nouzilly, France.
[7]Denotes equal contribution to the work presented.
[8]Denotes equal contribution to the project.

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Address for correspondence: Dr Colin Farquharson,
Division of Developmental Biology,
The Roslin Institute and R(D)SVS,
University of Edinburgh, Roslin,
Scotland EH25 9PS
UK
Tel: 44 (0)131 527 4399
Fax: 44 (0)131 440 0434
email: colin.farquharson@bbsrc.ac.uk

**ABSTRACT**

Bone strength is, in part, dependent on a mechanical input that generates a skeletal element of appropriate size and architecture to resist fracture during habitual use. However, the rate of longitudinal bone growth in juveniles can affect fracture incidence in late adulthood, suggesting an influence of early growth rate on later bone quality. We have compared the effects of fast and slow growth on bone strength and architecture in the tibiotarsi of embryonic and juvenile birds. The loading-related biochemical responses (intracellular G6PD activity and NO release) to mechanical load in these tibiotasal bones have been determined. Further, we have analysed the proliferation and differentiation characteristics of primary tibiotarsal osteoblasts from these fast and slow growing strains. Bones from chicks with divergent growth rates display equal resistance to applied loads, but when weight-corrected the data indicate that the bones from juvenile fast growth birds are weaker, with reduced stiffness and lower resistance to fracture. Cultured primary osteoblasts from slow-growing juvenile birds proliferated more rapidly, and had lower alkaline phosphatase activity. Bones from fast growing embryonic chicks display rapid radial expansion and incomplete osteonal infilling but, importantly, lack mechanical responsiveness. It is further evidence that the ability to respond to mechanical inputs is crucial to adapt skeletal architecture to generate a functionally appropriate bone structure. The mechanism of genetically-regulated fast growth in embryonic bone may predispose the bone to premature deformities and increased fragility in the adult.

## INTRODUCTION

It is a generally held view that an essential characteristic of a newly developed bone is the ability to adapt to changes in the local mechanical loading environment to prevent damage or failure [1, 2]. Mechano-adaptation during growth ensures that optimum bone mass and weight-bearing architecture are attained by the modelling and remodelling processes. Bone growth rate is a major determinant of these loading-induced mechano-adaptive modifications in bone (re)modelling [3-5]. Any imbalance in (re)modelling with subsequent increases in cortical bone porosity is associated with increased risk of fracture [6] and it is evident, therefore, that osteoporotic bone in the adult may represent a failure in the adaptive mechanism. Studies in humans demonstrate that the consequence of loading on bone mineral density and bone architecture is most influential prior to, and during, phases of most rapid growth [7-9]. Furthermore, Cooper and colleagues have reported that hip fracture risk in adulthood is greatest amongst those who were short at birth but then experienced a period of rapid 'catch-up' growth by 7 years of age [10]. It remains to be established whether there is a relationship between high growth rate, individual bone architectural characteristics and the ability to invoke mechano-adaptive responses.

One of the immediate responses to a period of osteogenic loading *in vivo* is an increase in intracellular glucose 6-phosphate dehydrogenase (G6PD) activity in resident osteocytes [11, 12]. Load-induced increases in bone cell G6PD activity are also evident in embryonic avian bones in culture [13] and is used as an early quantitative marker of cellular responses to applied load in bone tissue. Physiological levels of mechanical strain also promote rapid, transient and magnitude-related increases in nitric oxide (NO) release from bone explants and cultured bone cells *in vitro* [14-16]. The finding that mechanically-induced pre-osteogenic events are limited by non-selective inhibitors

of NO-synthase activity *in vivo* [17, 18] imply that load-induced adaptive changes are controlled, at least at their early stages, by NO production from the osteoblast/osteocyte network. It is therefore significant that these load-induced increases in intracellular G6PD activity and NO release are absent in bones from fast growth-selected, meat-type chick strains [19]. These data suggest that genetic selection for high growth rates is at the expense of mechano-adaptive mechanisms. Whether the lack of these early loading-related cellular responses from a young age predisposes skeletal elements to failure in the adult has not been considered previously.

Downstream mechano-adaptive responses to enhance fracture resistance can involve both periosteal accretion and a consolidation of the bone cortex. What remains unclear is whether there is a preferred architecture in mechanically non-responsive bones. Herein, we determine the architectural characteristics of bone, such as the degree of cortical porosity and periosteal expansion in bones from animals with differing growth rates, biomechanical properties and the capacity to exhibit appropriate responses to the application of mechanical stimuli. An association between cortical bone architecture and mechanical responsiveness will provide evidence for the hypothesis that these characteristics are linked.

This report evaluates these possible relationships in chicks with divergent growth rates generated by the selective breeding strategies. Many studies in commercial fowl have previously shown that the ability of bones to withstand deformities is often related to their inherent growth rate [20-23]. Thus, chickens which are the product of long-term selective breeding for high growth rate, suffer from growth-related skeletal abnormalities and bone deformities [24]. Therefore, we studied and compared tibiotarsi from three pairs of slow- and fast-growing birds (X44 and X33, SS and FS, and wild type

and meat type) to identify common responses, and ensure that they were not restricted to any one pair of chick strain. Particular variables were measured at both embryological and juvenile (post-hatch) time points in an attempt to cover the developmental windows that characterise rapid bone growth. It was envisaged that this approach would give a comprehensive insight into bone adaptation during growth, which involves the same fundamental mechanisms present in the adult skeleton but is more complex because of the interaction between ongoing adaptation and the underlying growth process. Accordingly, we measured various indices of bone architecture by histomorphometry in: i) embryonic and early post-hatch tibiotarsi from relatively distinct, unrelated chicks, selectively-bred for divergent growth rates for meat production (meat-type) compared with wild-type (wild-type) chicks, ii) compared tibiotarsi from closely-related, slow-growing and fast-growing genetically similar strains of chick at embryonic and early post-hatch stages (fast-growing, X33 and slow-growing, X44 strains; and, iii) another pair of genetically similar chicks at later post-hatch ages (fast strain, FS and slow strain, SS). We also calculated biomechanical properties and sought further evidence for reduced mechanical responsiveness in embryonic tibiotarsi with high inherent growth rate.

## MATERIALS AND METHODS

*Animals*

In this multi-centre study, two lines of paired fast and slow growing strains of chicks, each of an identical genetic background, were studied [25, 26]. One line had been selected through 32 generations for birds with high or low body weight at 8 weeks of age [25] and these referred to as X33 (fast-growing) or X44 (slow-growing). Another line comprised of a fast growing strain (FS), and a slow growing control strain (SS) that had not been subject to genetic selection since 1972 (Ross Breeders Ltd., Midlothian, UK; [26]. In addition, chicks from divergent genetic origin comprised Ross broilers, with high growth rate for meat production (Ross Breeders Ltd., Midlothian, UK; meat-type) compared with White Leghorn chicks (wild-type). All studies and protocols were approved by the Institutional Animal Users Committees of The Roslin Institute, University of Edinburgh and the Royal Veterinary College. Chicks from each line were killed, at the age specified, in accord with (UK) Animal Procedures act 1980. Post-hatch chicks were provided with water and fed *ad libitum*.

*Tissue collection and preparation*

Both left and right tibiotarsi were removed from at least 5 chicks of each strain (meat-type and wild-type, as well as X33 and X44), at each of the embryological or days post-hatch stages, and left bones processed as previously described [27]. Briefly, after fixation for 24 hours by immersion in 10% phosphate buffered formalin, the length of the left tibiotarsi was measured using Vernier callipers and the bone cut transversely at the mid-shaft using a low-speed, diamond-sintered annular saw (Microslice II; Cambridge Instruments, Malvern UK). Paired segments were dehydrated, cleared, methylmethacrylate-embedded [28], planoparallel sections cut from diaphyseal ends of each half and stained with 0.1% toluidine blue in 0.05M acetate buffer at pH 6.1 for analysis. Tibiotarsi from the

right limbs were stored at -20$^{O}$C and later used for the *ex vivo* strain calibration measurements, as a prelude to the experiments in which the effects of *in vitro* load application on bone cell activity and NO release were to be determined.

Tibiotarsi were removed from FS and SS chicks killed at 14 and 42-days-of-age (10 of each at each age) and a transverse segment from the mid-diaphysis of each was cut (as described above) and decalcified in 10% EDTA before embedding in wax. Toluidine blue-stained sections (7 μm in thickness) were analysed using 'Image Tool' for the determination of cortical porosity, osteon infilling (% of osteon occupied by newly elaborated bone) and osteocyte number/mm$^2$ within the newly elaborated bone in the primary osteon as previously described [26].

Additional FS (n=10) and SS (n=10) chicks were administered two intravenous calcein injections (10 mg/kg) 72 h apart at 18 and 21 days of age and killed 8 h after the last injection. A transverse segment from the mid-diaphysis of each tibiotarsus was cut and immersed briefly in 5% aqueous (wt./vol.) polyvinylalcohol (Grade GO4/140 Wacker Chemicals, Walton-on-Thames, UK) and chilled by precipitate immersion in *n*-hexane (BDH, Poole, UK; grade low in aromatic hydrocarbons) maintained at -70$^{O}$C and following removal stored at -70$^{O}$C (27). Frozen tissue was attached to the microtome chuck using 5% aqueous (wt./vol.) polyvinylalcohol. Unfixed, undecalcified sections were cut using a tungsten carbide-tipped steel knife (ARP, Cheshire, UK) in a Bright's cryostat (Bright Instruments, Huntingdon, UK). Sections (10μm) were flash-dried onto slides previously coated with 3-aminopropylsilane.

*Histomorphometry*

Using low power microscope objectives and a calibrated eyepiece graticule, the dorsoventral and mediolateral diameter of the tibiotarsal mid-shaft and of the medullary cavity were measured in each decalcified section (meat-type and wild-type, as well as X33 and X44) at the embryological stage or days post-hatch specified. The maximum width of the cortex was measured along four axes, each rotated by 90$^{o}$. Measurements of total osteonal bone area (% total area) and average width (μm), number and separation (μm) of osteonal walls (canal-canal distance) were made using methods described by Wright et al. [29]. Briefly, a digitising tablet coupled to a microscope system was used to draw around osteonal structures in each of four mid-cortical quadrants (caudal, ventral, lateral and medial) in sections from the mid-diaphysis of each tibiotarsus.

Frozen undecalcified sections of FS and SS chick bones were examined by fluorescence microscopy for the determination of the osteonal mineral apposition rate (MAR). Four images from each of two sections from each bone were analysed (along four axes previously described) using Image Tool. All areas examined were just inside the periosteal edge of the bone.

*Ex vivo strain measurement*

Loads were applied to isolated tibiotarsi (meat-type and wild-type, as well as X33 and X44) by means of a pneumatically operated actuator. To allow the same strain to be engendered, pressure-strain calibrations were performed for tibiotarsi from each type of chick. To do this, the cartilaginous ends were removed from the tibiotarsus leaving a central 9 mm segment of the bone mid-shaft from which the periosteum was removed exposing the bone surface, which was cleaned with diethyl ether. Two-four single-element, microminiature, strain gauges (EA-06-015-DJ; Measurements Group, Basingstoke UK) were bonded to different aspects at the mid diaphysis, with the gauge elements

aligned to the bone's longitudinal axis and protected with a silicone rubber coating. Each strain gauge was connected to an individual gauge conditioner and amplifier. Bone explants (with gauges attached) were transferred to the pneumatic loading apparatus and axially loaded at 1 Hz. at actuator pressures between 0.2 and 1.0 bar (up to 1.4 bar in older bones). The recorded profiles were used to determine dynamic peak strain magnitudes at increasing actuator pressures at the tibiotarsal mid-diaphysis of each chick type.

*Culture of embryonic chick bone and mechanical loading in vitro*

18 day-old X33 and X44 embryos were killed and tibiotarsi removed. Subsequently, the cartilaginous ends were removed aseptically, to leave a central 9 mm bone shaft segment, the marrow aspirated and adherent soft tissue and periosteum removed leaving the mid-shaft cortices and attached osteoblasts intact. After a brief wash in PBS, segments were cultured at the air/medium interface on grids for 5 hours, at 37$^{o}$C in a humidified 5% $CO_2$ incubator in Fitton-Jackson's modified BGJB medium supplemented with 2% FBS, 2 mM L-glutamine 50 μg/ml L-ascorbic acid and antibiotics (Life Technologies, Paisley, UK., [13]). After the 5-hour pre-incubation period, pairs of tibiotarsi were transferred to the loading apparatus, containing fresh medium, in which dynamic axial compressive loads were applied to one of each pair by a pneumatically operated actuator. Paired 'control' bones were held between polypropylene caps without dynamic loads applied and thus any extraneous vibrations were transmitted to both control and loaded bones. The applied load was adjusted (after calibration) to engender peak strain of -1300 to -1500 με (1 Hz. for 10 minutes, n=5) on the mid-shaft cranial surface of segments from either X33 or X44 strains. After loading, medium was collected, frozen and stored at -20$^{o}$C before measuring nitrite (stable NO metabolite) concentration and bones were immersed in PVA and chilled in *n*-hexane as described above.

*Assessment of glucose 6-phosphate dehydrogenase activity per cell*

Enzyme activity was measured as described previously [30, 31]. Fresh 10μm cryostat sections (see above) of each bone (X33 and X44) were incubated at 37$^{o}$C for 20 minutes in medium containing: glucose 6-phosphate (5mM, disodium salt; Sigma Ltd, Poole, UK); NADP (3mM, Roche Diagnostics Ltd, Lewes, UK) in 40% polypeptide P5115 in 0.05 M glycylglycine buffer at pH 8.0. Medium was saturated with nitrogen, and just prior to use, 3.7mM nitroblue tetrazolium (3.0 mg/ml, Sigma) was added. Sections were subsequently washed in water, dried and mounted in Aquamount (Sigma). Optical density of reaction product in individual osteocytes was measured with a Vickers M85A scanning and integrating microdensitometer [30]. At least 40 randomly selected osteocytes situated around the tibiotarsal mid-shaft near the periosteal bone surface were measured in each section, from at least 5 different bones in each assay and converted into mean integrated extinction (MIE x100) units/ 20 minutes.

*Measurement of nitrite concentration in culture medium*

Conditioned culture medium was assessed by chemiluminesence as described [32]. Briefly, appropriate volumes (10μl) of sodium nitrite solutions or sample supernatant (10-50μl) were injected via a septum into a reaction flask containing glacial acetic acid and sodium iodide at 85$^{o}$C, continually flushed with nitrogen, for conversion of nitrite ($NO_2^-$) to NO, which was drawn into a chemiluminesence analyser, where NO was quantified by its reaction with ozone [33]. NOS activity was confirmed by inhibition using either L-nitroso-arginine methyl ester (L-NAME; 1-100μM) or L-$N^5$-(1-Iminoethyl) ornithine (L-NIO; 10-100μM) added for 30 minutes prior to and during treatment.

*Osteoblast cell culture*

Four 21 day-old chicks of both the FS and SS strains were killed and both ends of the tibiotarsi, containing the epiphysis and metaphysis, were cut off and discarded and any adherent muscle or periosteum removed from the diaphyseal shaft using sterile swabs. The bone marrow was flushed using sterile PBS with 1% fungizone, and bone segments chopped into fragments and incubated at 37$^{o}$C on a shaker for 20 minutes in 4mls of 0.05% trypsin and 0.02% EDTA containing 0.6 mg/ml collagenase. Supernatant, together with 3ml of DMEM (without FBS) used to wash the bone fragments, was discarded. This was repeated 3 times but the supernatants from 3$^{rd}$ and 4$^{th}$ digestion retained together with DMEM used for washing, combined and after centrifugation the cells were cultured in DMEM containing 10% FBS and ascorbic acid (50 μg/ml). Second Passage cells were used for all experiments. Cells from both FS and SS were plated in multi-well plates at 20,000 cells/cm$^2$, with a minimum of 3 replicates. Osteoblast proliferation ($^3$[H]-thymidine uptake) was measured in pre-confluent cultures (day 3) and alkaline phosphatase activity assessed in post-confluent cells (day 11) according to Owen et al. (2007, [34]).

*Mechanical testing*

Bone stiffness and breaking strength were measured in tibiotarsi of 14 and 42 day old FS and SS chicks using a 3-point bending method. Briefly, tibiotarsi were cleaned and after bone length and width were measured, mechanical properties were measured using a 2500N load cell in a LRX materials testing machine (Lloyds Instruments, UK). Bone length and width were registered. 'Nexygen 2.2' software was used to measure load-to-failure in a specifically designed 3-point bending jig consisting of paired, curved lower pins, each of 10mm diameter spaced 30mm apart and a third pin of identical dimensions to apply a downward force (30 mm min$^{-1}$) halfway between the two

lower pins until the tibiotarsi was broken. The breaking strength was determined as the maximum load (N) to failure. Tibiotarsi stiffness (N/mm) was determined automatically from the slope of the load-displacement curve. To avoid introducing additional variation in the strength and stiffness measurements, each bone was orientated to ensure that bending occurred around the posterior-anterior axis. In addition, measurement of bone stiffness and breaking strength in tibiotarsi of 21 day old FS and SS chicks along with cross-sectional moment of inertia (CSMI, calculated using Scion Imaging Software and an in-house macro programme) were used to derive various bone biomechanical properties, including: ultimate stress, modulus and toughness [35, 36].

*Statistical analysis*

All results were reported as mean ± SEM. Comparisons were evaluated by Students t-test for unpaired samples on original numerical data. In all cases, $P < 0.05$ was considered statistically significant.

## RESULTS

*Porotic bone cortices are a persistent characteristic of chicks selected for high growth rate*

Previous data from the cortical bone of X33 and X44 line of chicks [37] had established that genetic selection for high rates of growth was associated with porotic bone architecture. Cortical morphometric analysis of the tibiotarsi of fast (FS) and slow (SS) growing chicks showed that cortical porosity within the periosteal/endosteal envelope was higher in FS compared to SS chicks at both 14 and 42 days of age (Figure 1a). This indicates that the skeletal architecture of chick bone from strains genetically selected for fast growth is more porous.

To examine whether other bone architectural traits are evident at the pre-hatch and early post-hatch stages in distinct lines of chick selected for fast growth rate we measured; weight, tibiotarsal length, average width, number, area and separation of osteonal walls at the diaphyseal tibiotarsal mid-shaft in chicks selectively bred for high growth rate (meat-type) and white leghorn chicks (wild-type). This confirmed that meat-type chicks were significantly heavier than the wild-type strain at embryonic (14 and 18 days after fertilization, pre-hatch) and post-hatch stages (Figure 2a). This was accompanied by an increased tibiotarsal length in meat-type chicks only at embryonic day 14 (Figure 2b.). Evaluation of bone distribution at the mid-diaphysis showed that osteonal wall widths and area were similar in wild-type and meat-type chick bones. There was, however, a significant reduction in osteonal wall number as well as an increase in wall-to-wall separation (canal diameter) in meat-type compared to wild-type chicks (Figure 2c-f). Together with results presented in Figure 1 and previously published findings [19], these data (Figure 2) indicate that all three of these distinct strains of bird (FS and meat-type herein, and X33), each the product of independent selection for fast

growth, show enhanced cortical bone porosity. One possible modelling design by which this could be achieved is a lag between periosteal expansion and the rate of infilling of newly established osteons.

*Diminished osteonal infilling and MAR without changes in osteocyte density in high growth rate chicks.*

An explanation for the increased porosity in the FS strain of chicks was therefore sought by comparing the amount of osteonal infilling in cortical bone of juvenile FS and SS chicks. These investigations disclosed a decrease in the infilling of the primary osteons in the periosteal region of the FS chicks at both 14 and 42 days of age (Figure 1b). Osteon area within the periosteal region was similar in FS and SS chicks at both 14 and 42 days of age (Figure 1c). Together these data suggest that osteoblast activity within the osteons of the fast-growing FS chicks was significantly lower than in slow-growing SS chicks. This was confirmed by measurements of MAR which were significantly lower ($P<0.001$) within periosteal located osteons of the juvenile FS compared with slow-growing SS chicks (Figure 1d). This measurement was made in 21-day-old chicks, which we regarded as a representative age of a rapidly growing skeleton and therefore likely to mirror the developmental adaptations observed in the 14 and 42 day-old birds. This decreased MAR was not, however, associated with altered rates of osteoblast-osteocyte transition during incorporation into the matrix, since osteocyte density was unchanged within the newly elaborated bone in the primary osteons at both 14 and 42 days of age (Figure 1e). These results indicate that increased bone porosity is a conserved trait in chick lines that is the product of selective breeding for fast growth and is achieved by enhanced rate of new periosteal osteon formation coupled with deficient infilling.

*Bone load-bearing competence is modified in fast growth rate chicks*

To determine whether these conserved porotic architectural traits result in functionally modified bone strength we measured bone stiffness and breaking strength in tibiotarsi of juvenile FS and SS chicks. We found that tibiotarsal bone stiffness was higher in the FS than in SS chicks at both 14 days and 42 days (Figure 3a). We used bodyweight as a covariate as a strong linear relationship has been shown to exist between log (CSMI) and log (body weight) [18]. Therefore, adjustment for body weight resulted in a 'corrected' bone stiffness that was significantly lower in the FS chicks than SS chicks at 14 days (FS = 60 ± 0.7 N/mm, SS = 99 ± 0.6 N/mm; $P < 0.01$). By 42 days of age, however, no significant difference was evident between the strains after adjustment (FS = 363 ± 1.8 N/mm, SS = 361 ± 1.1 N/mm). Tibiotarsal breaking strength was also higher in FS chicks at 14 days with no significant difference at 42 days. By contrast, adjustment for body weight disclosed a breaking strength that was lower in FS chicks both at 14 days (FS = 63 ± 1.4 N, SS = 83 ± 1.2 N; $P < 0.01$) and at 42 days of age (FS = 296 ± 2.3 N, SS = 362 ± 0.5 N; $P < 0.01$). Thus, tibiotarsi of faster-growing FS chicks exhibit both reduced breaking strength and stiffness than slower-growing SS chicks when a correction for body weight was applied. These findings were strengthened by calculation of biomechanical properties based on bone morphometry (CSMI, ultimate stress, modulus and toughness; see Table 1) which showed, prior to correction for body weight, that modulus and ultimate stress were higher in FS than in SS chicks at 21 days, and that after weight correction tibiotarsi of faster-growing FS chicks exhibit reduced ultimate stress, modulus and toughness than slower-growing SS chicks.

*Morphometric differences at the tibiotarsal mid-diaphysis in genetically-related fast/slow growing chicks*

In mechanical terms, changes in bone breaking strength and stiffness can be achieved by modifying the CSMI; depositing bone mass at greater distances from the centroid [38]. To determine whether selection for fast growth is associated with the distribution of bone mass further from the centroid, we examined changes in tibiotarsal diameter, medullary diameter and cortical thickness in meat-type, and wild-type bones, as well as in X33 and X44 chick bones at various stages pre-and post-hatch. We found that cortical diameter (total width) was greater in meat-type than wild-type tibiotarsi at all times examined and that similar trends were evident when X33 and X44 chick bones are compared (Figure 4a-b). In all strains, the diameter of the medullary cavity exhibited an almost constant linear rate of expansion (Figure 4c-d); but whilst this rate was similar throughout X33 and X44 chick bone development there was a post-hatch divergence in the rate of medullary expansion between meat-type and wild-type chicks (Figure 4c-d). Our results also indicate that the cortical width is diminished at later post-hatch time points in all strains of chicks, but that this remains greater in faster growing (meat-type and X33) chick strains (Figures 4e-f). It is clear from these measurements that in addition to an increased radial expansion of the bones in fast growing, X33 and meat-type chicks that there is also an increase in the width of the cortices compared to X44 and wild-type chicks, respectively. These data are consistent with the notion that fast growth is invariably associated with enhanced radial expansion, by faster addition of new osteons at the perimeter in these chicks. It is possible that this reflects adaptation to the greater loads engendered by virtue of the higher body weight. Indeed, it has previously been shown that feed-restriction results in reduced tibial width (almost to control levels) and lower porosity in FS chicks, suggesting that the increased periosteal expansion (and increased porosity) is the product of bone responses to heavier body weight in faster-growing chicks

[18]. An alternative explanation of our findings is that the distinct bone architecture in these birds is not due to adaptation to higher body weight as suggested by others [29], but due to intrinsic differences in osteoblasts in these faster growing birds, predisposing them to reduced osteonal infilling even in feed-unrestricted conditions.

*In vitro osteoblast proliferation and, differentiation differ in FS chicks*

To examine whether bone cells in fast growth chicks have intrinsic differences in their behaviour or are subject only to *in vivo* systemic regulatory controls, we have sought evidence that osteoblasts derived from juvenile FS bones exhibit differences in proliferation and differentiation when compared to those derived from SS chick bones. We found that proliferation after 3-days in culture was higher in osteoblasts from the slow growing birds (Figure 5a) whereas ALP activity after 11-days in culture was significantly lower (Figure 5b).

*Fast growth embryo tibiotarsi show reduced load-induced osteocyte G6PD activity and NO release*

Previous findings have suggested that selection for high growth rates compromise the mechanoadaptive response of bone [19]. Amongst the earliest *in vivo* and *in vitro* indices of this response are increases in bone cell G6PD activity and NO release. Using a well-established model for applying controlled mechanical loads to embryonic chick tibiotarsi, we have therefore examined for the first time whether these indices are modified in tibiotarsal bones from X33 and X44 chicks at 8 weeks of age. As a prelude, we first calibrated the *in vitro* loading of these X33 and X44 tibiotarsal bones, by measuring load-strain relationships, on both the caudal and cranial surfaces and compared these to those measured in wild-type and meat-type 18-day old embryos. We found that strain magnitude increased linearly with increasing actuator pressure and that engendered strains were

consistently greater on cranial than on caudal surfaces (Figure 6a-b). For an applied pressure of 0.6 bar, the dynamic peak compressive strain magnitudes (caudal/cranial bone surfaces) were similar, namely: -550/-1500 and -1000/-1250με in wild-type and meat-type chicks, respectively (Figure 6a-b). We also found that the strain magnitude increased with increasing applied actuator pressures between 0.2 and 0.8 bar in both 18-day embryonic X33 and X44 tibiotarsi (Figure 6c-d). Tibiotarsi from 5-day post-hatch X33 and X44 chicks showed almost identical patterns of changing strain magnitude at the tibiotarsal midshaft with increasing applied actuator pressures between 0.3 and 1.4 bar (data not shown). At this age, there were significant increases in the tibiotarsal load resistance at comparable actuator pressures to those evident in 18-day embryonic tibiotarsi. Thus, for an applied pressure of 0.6 bar the dynamic peak compressive strain magnitudes (on caudal/ cranial bone surfaces) were similar in these tibiotarsi, namely: -360/-600 and -340/-540 με in X33 and X44, respectively (data not shown).

Having calibrated the loading of bones, we subsequently exposed bones from X33 and X44 lines to identical strain magnitudes in an organ culture system in order to examine recognised marker indices of bone cell responsiveness, namely osteocyte G6PD activity and NO release. We found that application of load to X44 embryonic tibiotarsi for 10 minutes produced significant increases in osteocyte G6PD activity (Figure 7a). In contrast, load application to X33 tibiotarsi, engendering identical peak strain magnitudes produced smaller, statistically insignificant (Figure 7a) increases in osteocyte G6PD activity. Comparison of nitrite accumulation in medium conditioned by tibiotarsi showed that the X44 strain exhibited statistically significant increases in NO release in response to load application ($p<0.05$), but that in contrast X33 chick bones failed to exhibit any modification in load-induced NO release (Figure 7b).

## DISCUSSION

Few studies have addressed the effect of growth rate on the architecture, biomechanical properties and behaviour of skeletal elements. However, collectively those that have imply a primary influence of bone structure [39, 40]. We agree with this conclusion, but our findings suggest, additionally, that selection for a higher growth rate within in a species can also result in a modified skeletal architecture. This study has compared the tibiotarsal bones of three different strains of fast-growing chick and tibiotarsi from both closely-related as well as unrelated, slow-growing birds. These studies might help explain why commercially-bred chickens, which are the product of long-term selective breeding for high growth rate (particularly of muscle mass), suffer from an increased incidence of cortical bone deformities [41, 42]. In this study we have described the differences in mechanical properties, bone architecture (bone size and matrix distribution), and the response to applied mechanical loading. It remains a possibility that the timing of peak growth velocity and maturation rates may differ in chick strains. For example, a slow increase in mineralisation after 42 days could improve the mechanical properties of the skeletons dramatically, while changes in cortical wall thickness might bring the groups together. Further, in the mechanical testing studies, the rate of displacement will influence the measured stiffness of bone and for this reason further study using rates of displacement lower than those selected herein (30 mm $min^{-1}$) may also be informative. These variables could impact on our conclusions. Regardless, we demonstrate an association between growth rate, architecture and mechanical responsiveness of the resident bone cells in embryonic and juvenile individuals.

*Ex vivo* comparison of the bones of the genetically divergent wild-type and meat-type birds disclosed no difference with respect to their load/strain curves. This was also the case for the more closely related X33 and X44 birds and thus, the weight-independent data illustrates that the bones from birds with different growth rates have, in fact, similar load-bearing capacity. Uncorrected biomechanical properties indicate that the bone quality is the same, but the CSMI differ, suggesting differing architectures. In fast-growing birds, the tibiotarsi consistently display greater bone cortical diameters and the more rapid addition of new osteons at the periosteal perimeter. In these strains, the increased diameter is coupled to differences in the number, width and positioning of the bony struts compared with slow-growing birds. This results in a cortical shaft that displays significant increases in porosity in the fast-growing birds. However, the weight corrected biomechanical data indicates bones from juvenile fast growth birds are weaker, with reduced stiffness and lower resistance to fracture.

It has been postulated that resistance to fracture in limb bone is by adaptation to achieve a target load-related strain level [43]. Fast growth results in a skeletal architecture which achieves strength independent of adaptation to loading. Although this is in agreement with previous studies [24], it is unknown whether the morphological characteristics of the rapidly growing bone may ‘catch-up’ once ossification of the epiphyseal plates is complete. Further studies where tibiotarsal longitudinal growth has achieved a plateau would, therefore, be useful.

Our findings indicate also that the distinct architectures may be at least partly due to intrinsic differences in the osteoblastic population in these faster growing birds. We found that isolated osteoblasts derived from the fast-growth chicks proliferate at a slower rate but differentiate more readily when compared directly to closely-related slow growing birds. The relevance of these

findings are yet to be defined, but imply that the behaviour of isolated osteoblasts derived from fast and slow growth strains are independent of local environmental (culture) cues and that the osteoblasts from fast and slow growth birds are inherently different.

In our attempt to ensure that the results obtained were of a generic nature and not strain-specific we compared a number of complimentary parameters from three different pairs of fast- and slow-growing birds. Further, we analyzed some variables at both embryological and post-hatch time points in an attempt to cover three windows characterized by rapid bone growth. It is accepted that in order to achieve these goals certain compromises were made *i.e.* all methods were not applied to all strains and at all developmental ages, however, we believe that such compromises were justified and consistent with our overall aims to understand more fully the effects of rapid growth on bone architecture and response to mechanical loading. It is clear, nonetheless, that our studies are limited to birds that have yet to reach full maturity and are restricted to a single bone type. Extrapolation to different bones in other species should, therefore, be made with caution.

Our study is also consistent with previous reports detailing a relative failure of bones from birds with fast-growing attributes to respond to applied mechanical loads *in vitro* organ culture [19]. This is exemplified by the restricted load-induced increases in intracellular G6PD activity and NO release found in the tibiotarsi of faster growing chicks. Although the full relevance of such load-induced increases in G6PD activity have yet to be established fully *in vivo*, studies in which NO production was blocked by administration of selective NO-synthase inhibitors in rodent loading models have demonstrated the essential role played by NO production during the mechanotransduction phase of

these adaptive responses [17, 18]. Both NO release and G6PD activity have been shown to increase more markedly in response to mechanical loading in wild-type and egg-laying bird tibiotarsi [19].

We have described inherent architectural and biochemical differences in the bones from wild-type, fast and slow growth chickens. We suggest that the tibiotarsi of faster growing birds achieve mechanical competence without reference to the prevailing mechanical environment, and represents an 'elemental' mechanism of bone growth - resulting in a self-limiting structure. This mechanism is satisfactory up to a point, but the lack of osteonal infilling in the long-term is likely to predispose these bones to premature failure. Taken together, our data suggest that genetic selection for fast growth generates bone architectures in which increases in new bone surfaces through enhanced periosteal expansion are at the expense of cortical consolidation, and that these features are apparently either inextricably linked to, or co-selected with, diminished early responses to mechanical loading. This may help to explain the differences in the amount of bone formed in the giant composite canals seen in cases of hip fracture compared to that in the more normal "simple" canals [44]. Furthermore, our in vitro findings support the possibility that these bone characteristics may be underpinned by inherent and autonomous changes in osteoblast behaviour. The notion that increases in bone porosity are in some way linked to modified sensitivity to mechanically-induced changes in cell behaviour is novel and has many implications for our understanding of the factors that predispose bone to increased fragility and deformities.

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**FIGURE LEGENDS**

**Table 1. Calculated biomechanical properties of bones from fast- and slow-growing are the same, but altered when corrected for body weight.** CSMI, ultimate stress, modulus and toughness were calculated based on bone architecture and deformation tests. The CSMI for slow growing birds is significantly smaller than that for fast-growing birds. However, this difference is lost when the data are corrected for body weight. In contrast, whilst there is no difference for ultimate stress, modulus and toughness in the data, suggesting similar bone qualities, significant differences are apparent when the data are weight corrected.

**Figure 1. Cortical bone in post-hatch fast-growing chicks is more porous and exhibits reduced mineral appositional rate and osteonal infilling.** Bone architecture was analysed in transverse sections of the mid-diaphyseal region of the tibiotarsi from fast (FS) and slow (SS) growth strains of chick at 14 or 42 days of age as described. Cortical bone of FS chick tibiotarsi exhibited greater cortical porosity (a), reduced osteon infilling (b) without any modification in osteon size (c) or osteocyte density (e) than SS chicks at both 14 and 42 days of age. Analysis of mineral appositional rate (d) in transverse sections of the mid-diaphyseal region from FS and SS strains of chick at 21 days of age demonstrated a reduced rate of mineral apposition on cortical bone surfaces between 18 and 21 days in the FS chicks. Data are shown as mean ± SEM and * denotes $P<0.05$; ** $P<0.01$; ***$P<0.001$.

**Figure 2. Cortical bone in embryonic meat-type chicks is more porous than in wild-type chicks.** Analysis of bone organisation was evaluated in transverse sections of the mid-diaphyseal region of embryonic and post-hatch tibiotarsi from chickens commercially-selected fast-growth for meat

production (Meat-type) compared to Wild-type slower growing strains. Total body weight (a) was greater in Meat-type than in Wild-type chicks at all embryonic and post-hatch stages examined, whilst at the time points studied tibiotarsal length (b) was only elevated in the Meat-type versus the Wild-type chicks at 14 days post-fertilisation. Enhanced porosity of the Meat-type chick cortex was evident in the reduced density of osteons (d) and greater canal-canal distances (e), without modification in bone area (c) or osteonal wall widths (f) when compared to Wild-type chicks. Results are presented as mean ± SEM (NS; not significant) and * denotes $P<0.05$ and ** $P<0.01$

**Figure 3. Differences in load-bearing properties in tibiotarsi of fast- and slow- growth rate chicks.** *Ex vivo* measurements of post-hatch tibiotarsal bone load-bearing competence demonstrates that the slower growth strain (SS) of chick exhibits lower bone stiffness (a) than FS chicks at both 14 and 42 days of age and lower tibiotarsal bone breaking strength than FS chicks at 14 days but not 42 days post-hatch. The effect of correction for body weight is discussed. Data are shown as mean ± SEM, *** denotes $P<0.001$.

**Figure 4. Morphometric differences in mid-diaphyseal tibiotarsal architecture in embryonic and post-hatch genetically-diverse (Meat-type versus Wild-type) and genetically-related (X33 and X44) fast and slow growth rate chicks.** Total width (a and b, top pair), medullary diameter (c and d, middle pair) and mean width of the cortex (e and f, bottom pair) of the tibiotarsal bone of Meat-type and Wild-type (a, c and e, left), and X33 and X44 (b, d and f, right) strains of chick at 14 and 18 days post-fertilisation and at 2 days post-hatch support the contention that fast growth is invariably associated with enhanced radial expansion. Data are shown as mean ± SEM, * denotes $P<0.05$ and ** $P<0.01$, respectively.

**Figure 5. Osteoblasts derived from the cortical bone of fast-growing and slow-growing chicks exhibit intrinsic differences in their behaviour *in vitro*.** Proliferation rates (a, thymidine incorporation, dpm) in osteoblasts derived from the cortical bone of FS chicks are diminished relative to those observed in osteoblast-like cells from SS chick bone at a pre-confluent stage of incubation (day 3). Alkaline phosphatase activity (b, pNPP hydrolysed/unit time/mg protein) in osteoblasts derived from the cortical bone of FS chicks are enhanced relative to those observed in osteoblasts from SS chick bone at a post-confluent (day 11) incubation stage. Data are shown as mean ± SEM, *** denotes $P<0.001$.

**Figure 6. Calibration of load-strain relationship in 18 day old embryonic (post fertilisation) tibiotarsal bones from Wild-type, Meat-type, X33 and X44 chick strains.** Measurements of mechanical strain (με) at different actuator pressures on the caudal and cranial surfaces of the mid-diaphyseal bone in genetically-divergent Wild-type (a) and Meat-type (b), and on the cranial aspect of genetically-similar X33 (c) and X44 (d) 18 day embryonic chick tibiotarsi.

**Figure 7. Restricted genetic selection (X33 versus X44) for fast growth modifies load-induced changes in bone cell behaviour and nitric oxide release.** Mechanical load (10 minutes) application engendering similar peak strain magnitudes induces significant increases in (a) osteocyte glucose 6-phosphate dehydrogenase activity (mean integrated extinction, MIE x100/cell/20 minutes) and (b) the accumulation of nitrite in the medium (μM; mean±SEM) from tibiotarsal bone segments from X44 (slow-growth), but not X33 chick strains maintained *in vitro*. Data is expressed as mean ± SEM, * and ** denote significance at a level of $p<0.05$ and $p<0.01$, respectively (n=4 bones in each

experiment; triplicate sections for each bone analysed for changes in G6PD activity, triplicate experiments for each type of chick).

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Table 1

| | Uncorrected | | Body Weight Corrected/g | |
|---|---|---|---|---|
| | **Fast** | **Slow** | **Fast** | **Slow** |
| CSMI ($mm^4$) | 42.77 ± 2.86 | 24.91 ± 2.70*** | 0.08 ± 0.01 | 0.09 ± 0.01 |
| Ultimate Stress (MPa) | 520.72 ± 20.81 | 544.27 ± 16.62 | 0.10 ± 0.05 | 1.95 ± 0.10*** |
| Modulus (GPa) | 1.49 ± 0.08 | 1.76 ± 0.09* | 0.003 ± 0.001 | 0.006 ± 0.001*** |
| Toughness ($MJ/mm^3$) | 328.61 ± 24.72 | 292.75 ± 18.50 | 0.63 ± 0.06 | 1.06 ± 0.09*** |

- $p<0.05$ vs Fast ***$p<0.001$ vs Fast

Figure 1

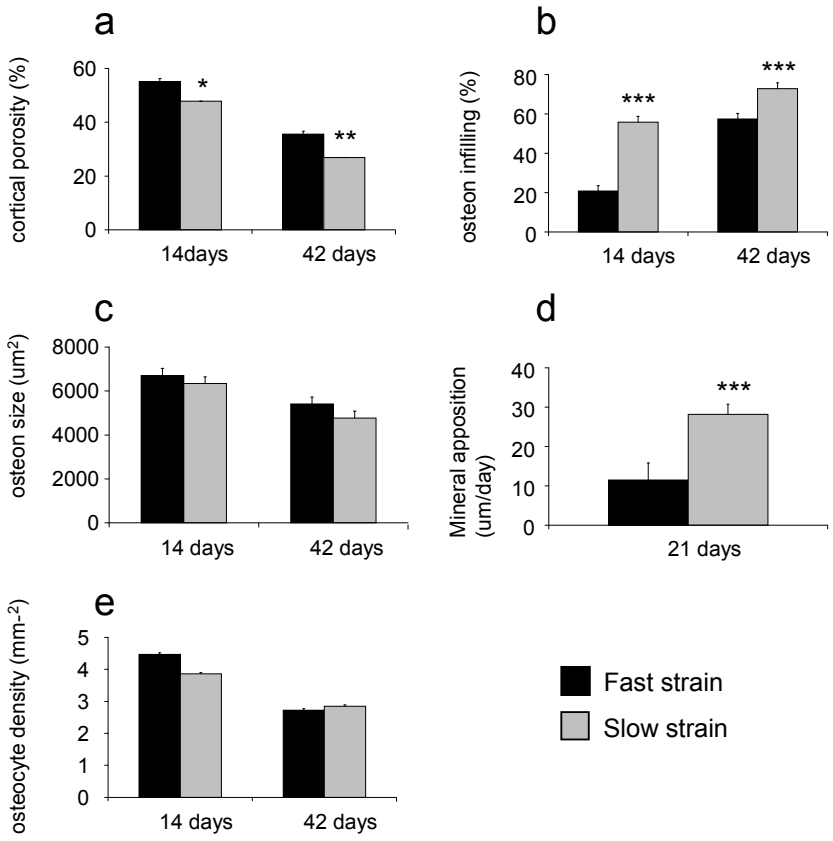

a
cortical porosity (%)
60
40
20
0
*
**
14days
42 days
b
osteon infilling (%)
80
60
40
20
0
***
***
14 days
42 days
c
osteon size (um2)
8000
6000
4000
2000
0
14 days
42 days
d
Mineral apposition (um/day)
40
30
20
10
0
***
21 days
e
osteocyte density (mm-2)
5
4
3
2
1
0
14 days
42 days
Fast strain
Slow strain

Figure 2

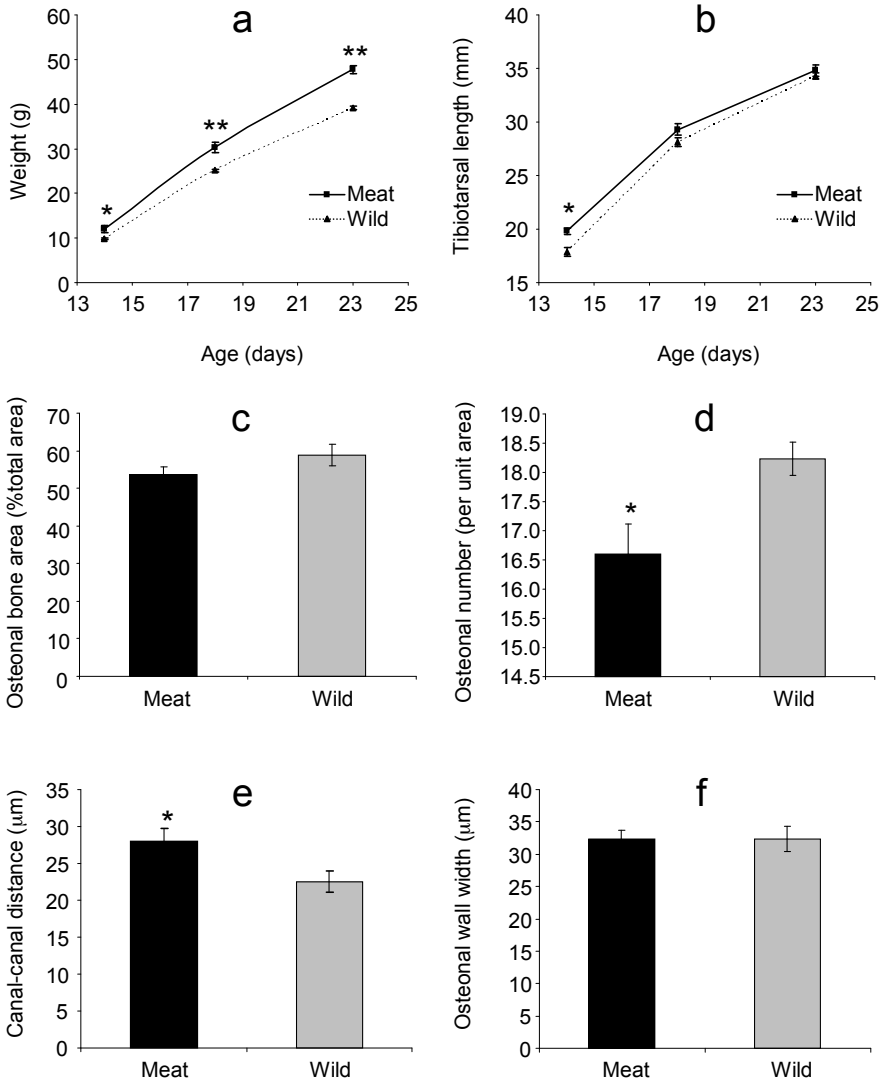

a
Weight (g)
Age (days)
Meat
Wild
b
Tibiotarsal length (mm)
Age (days)
Meat
Wild
c
Osteonal bone area (%total area)
Meat
Wild
d
Osteonal number (per unit area)
Meat
Wild
e
Canal-canal distance (μm)
Meat
Wild
f
Osteonal wall width (μm)
Meat
Wild

Figure 3

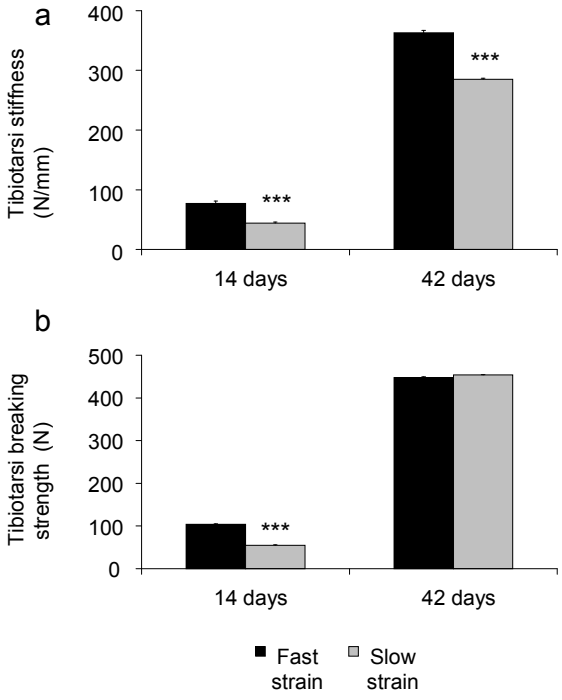

a
Tibiotarsi stiffness (N/mm)
400
300
200
100
0
***
***
14 days
42 days
b
Tibiotarsi breaking strength (N)
500
400
300
200
100
0
***
14 days
42 days
Fast strain
Slow strain

Figure 4

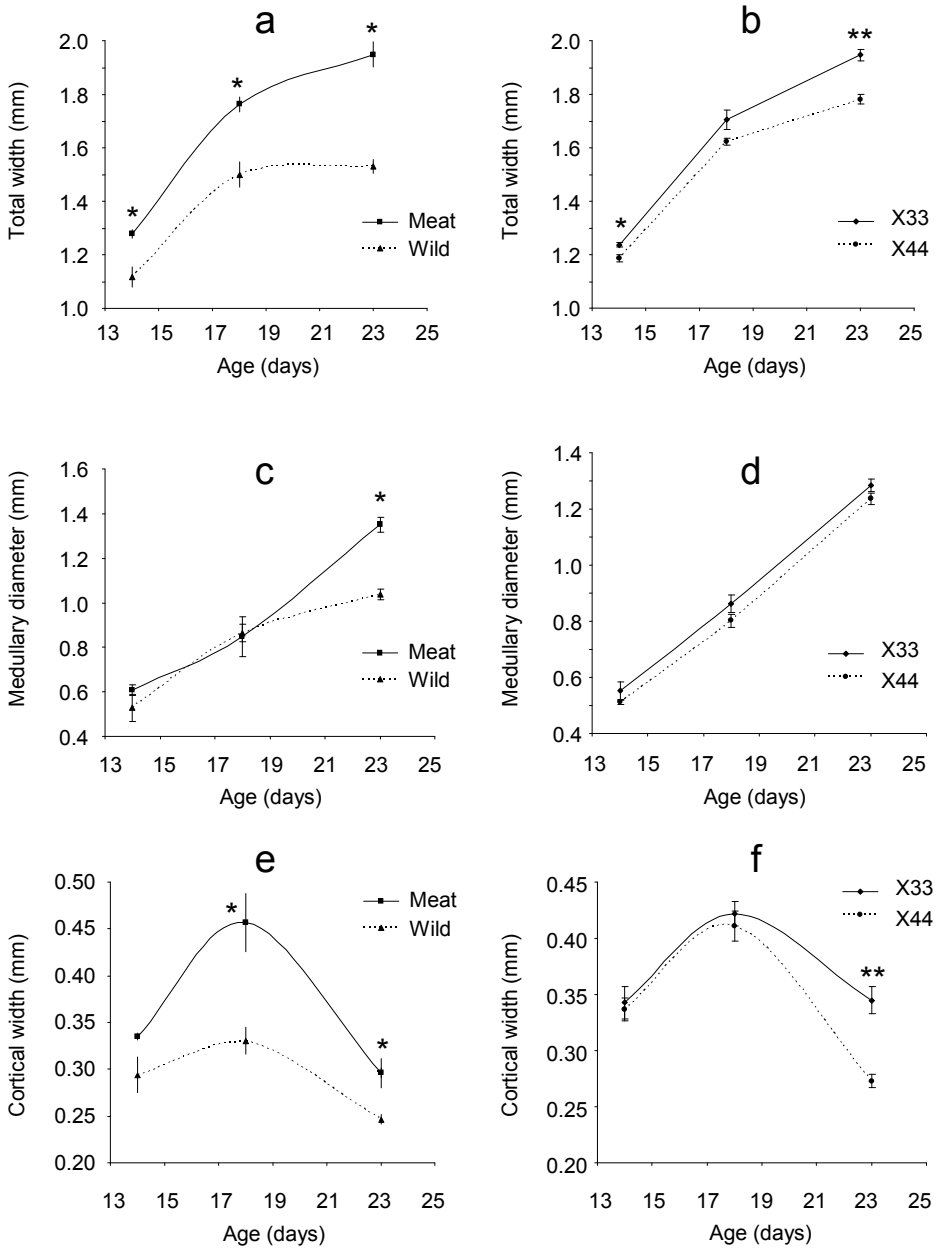

a
b
c
d
e
f
Total width (mm)
Medullary diameter (mm)
Cortical width (mm)
Age (days)
Meat
Wild
X33
X44
*
**

Figure 5

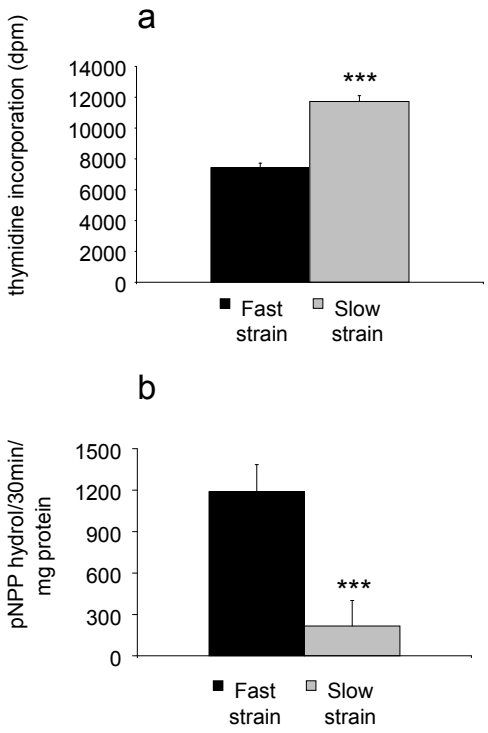

a
thymidine incorporation (dpm)
14000
12000
10000
8000
6000
4000
2000
0
***
Fast strain
Slow strain
b
pNPP hydrol/30min/ mg protein
1500
1200
900
600
300
0
***
Fast strain
Slow strain

Figure 6

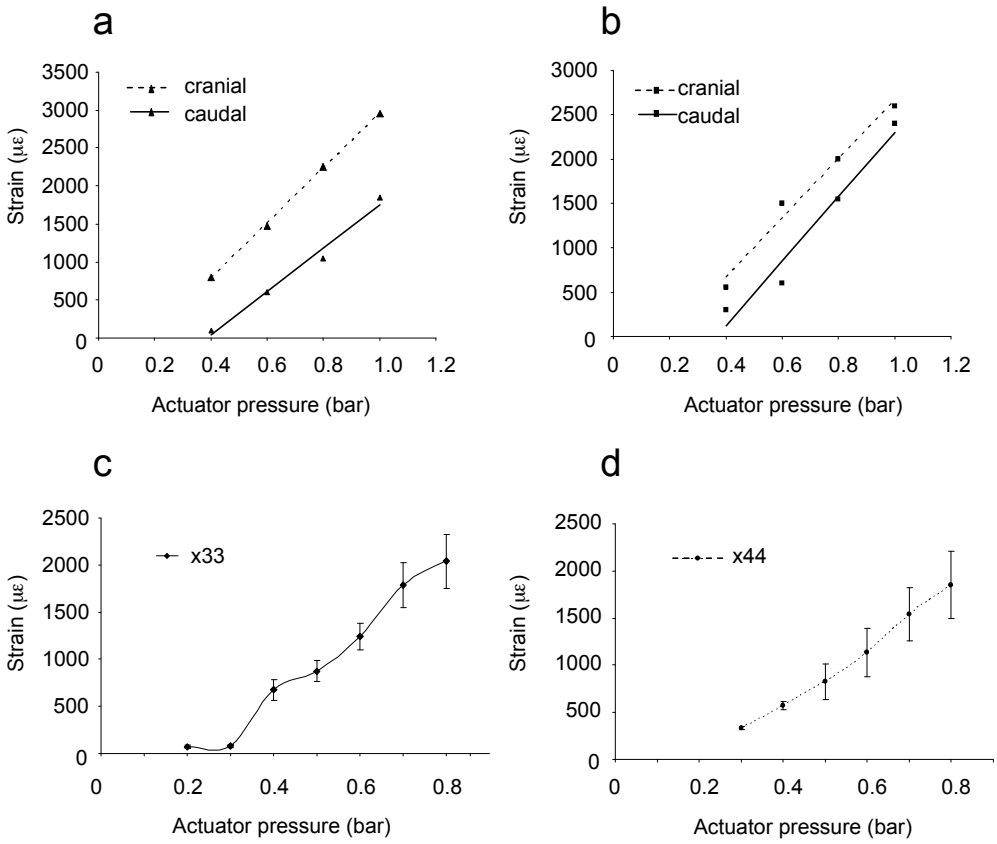
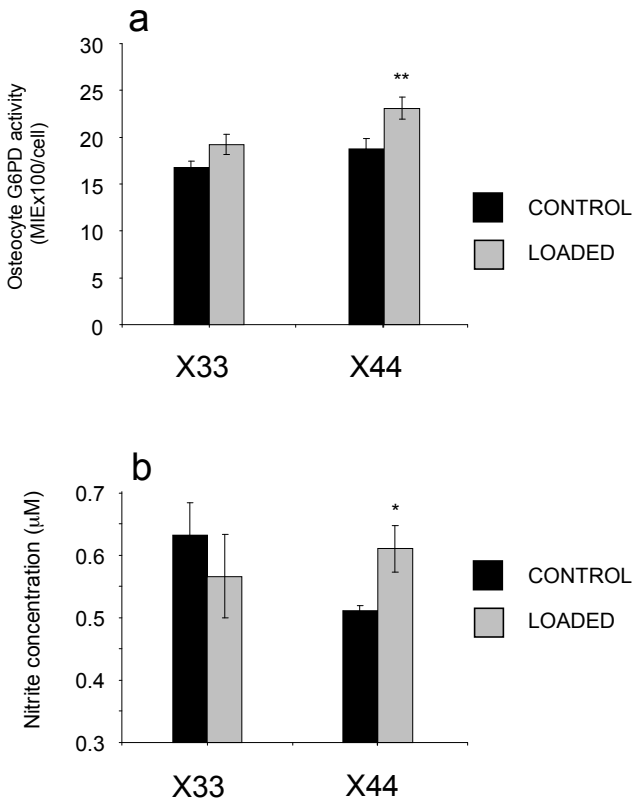

a
cranial
caudal
Strain (με)
Actuator pressure (bar)
b
cranial
caudal
Strain (με)
Actuator pressure (bar)
c
x33
Strain (με)
Actuator pressure (bar)
d
x44
Strain (με)
Actuator pressure (bar)

Figure 7


a
Osteocyte G6PD activity (MIEx100/cell)
30
25
20
15
10
5
0
**
X33
X44
CONTROL
LOADED
b
Nitrite concentration (μM)
0.7
0.6
0.5
0.4
0.3
*
X33
X44
CONTROL
LOADED